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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> Heparanase (HPR1) is an endoglycosidase that specifically degrades heparan sulfate proteoglycans, a main constituent on the cell surface and in the extracellular matrix and basement membrane. Previous studies using xenograft models have shown that increased HPR1 expression can promote tumor growth. Interestingly, recent studies suggest that HPR1 may promote tumor growth independent of its enzymatic activity because of the C-terminus of HPR1 can activate the PI-3 kinase pathway. Studies during the current grant period revealed that sulodexide, a HPR1 inhibitor, stimulate breast tumorigenesis and tumor growth of polyoma virus middle T antigen-induced breast tumor in a somatic and syngeneic breast cancer models. To further explore the role of HPR1 in breast tumorigenesis and growth, we knocked down heparanase in a breast cancer cell line derived from mice infected with the vector encoding the Neu oncogene. We found that HPR1 knockdown led to a significant reduction of tumor growth in a syngeneic mouse model and delayed tumor growth in a somatic mouse model. Our results suggest that HPR1 may promote tumor growth independent of its enzymatic activity.					
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## Introduction

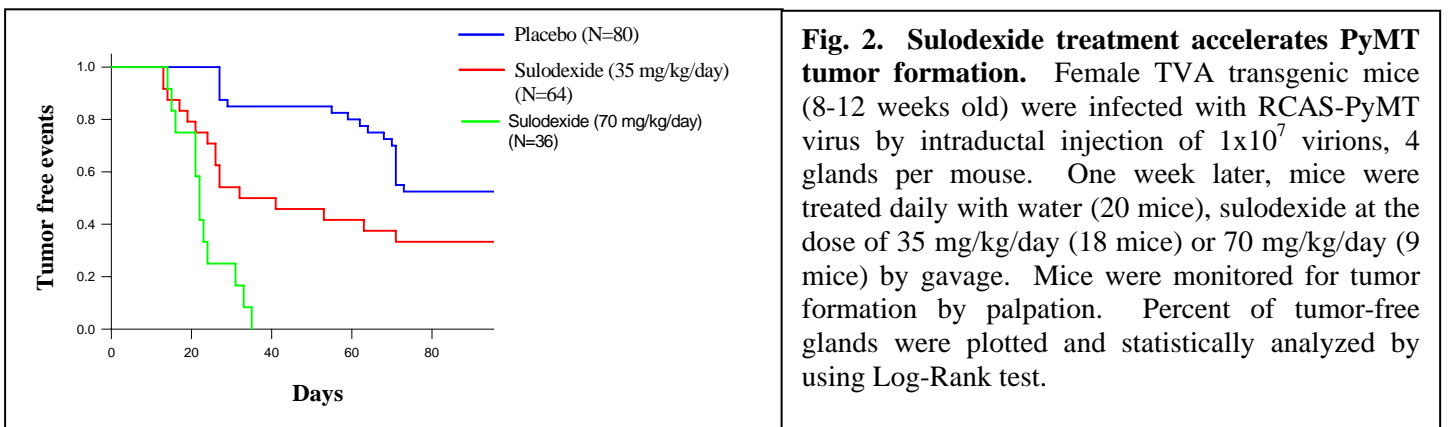
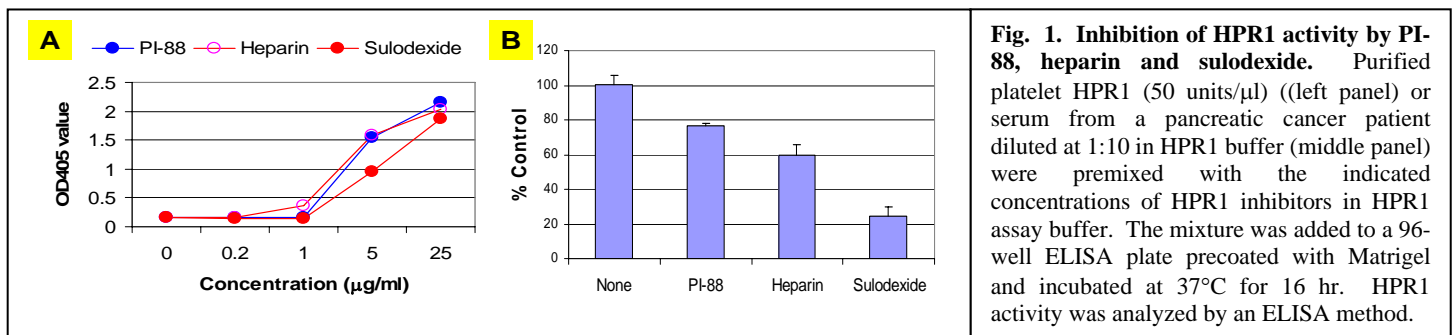
Heparanase-1 (HPR1) is an endoglycosidase overexpressed in many malignancies including breast cancer (1; 2). Previous studies suggest that the enzymatic activity of HPR1 can promote tumor angiogenesis and growth by degrading extra cellular matrix and releasing the growth factors. Since the C-terminus of HPR1 can activate the PI-3 kinase pathway and induce endothelial and tumor cell migration independent of its enzymatic activity, it is not clear whether its enzymatic activity or C-terminus or both contribute to breast tumor initiation and growth. The goal of this project is to dissect the opposing effect of HPR1 enzymatic activity and HPR1 C-terminus epitope on breast tumor initiation in a clinically relevant mouse breast cancer model. We proposed to determine if HPR1 knockdown will suppress or accelerate breast tumor initiation mediated by three oncogenes, PyMT, Neu and Wnt, and whether HPR1 C-terminus or an enzymatically dead HPR1 can stimulates breast tumor initiation, whereas full-length HPR1 has no effect or is less effective in stimulating breast tumor initiation and progression.

## Experimental procedures and results

**Sulodexide treatment accelerates PyMT-mediated tumorigenesis.** Sulodexide is a mixture of dermatan sulfate (20%) and low-molecular-weight heparin (80%) (Keryx Biopharmaceuticals, Inc., New York). We examined the ability of sulodexide to inhibit HPR1 activity by using a novel ELISA method developed in my laboratory (3-7). As shown in Fig. 1 (left panel), sulodexide inhibited HPR1 activity with an IC<sub>50</sub> value of approximately 5 µg/ml. The IC<sub>50</sub> values for heparin and PI-88 were approximately 2-3 µg/ml. Sulodexide inhibits HPR1 activity slightly better than PI-88 and heparin when a pancreatic cancer patient's serum was used as the source of HPR1 (Fig. 1, right panel).

We next tested whether sulodexide can prevent breast tumor formation. TVA transgenic mice were infected by intraductal injection of RCAS-PyMT virus,  $1 \times 10^7$  virions/gland, 4 glands/mouse. Mice were treated with water or sulodexide at the dose of 35 or 70 mg/kg/day by gavage and monitored for tumor formation by

palpation. To our surprise, administration of sulodexide accelerated breast cancer formation in a dose-dependent manner (Fig. 2). Breast tumors were formed in untreated mice with the median latency of  $>78\pm4.1$  days, whereas administration of sulodexide at 70 and 35 mg/kg/day had a median tumor latency of  $23\pm2$  days and  $53\pm7.4$  days, respectively. Log-rank test showed that sulodexide treatment at the dose of 35 mg/kg/day significantly shortened tumor latency, compared to the untreated control group ( $p=0.018$ ). Also, Log-Rank test revealed that mice treated with sulodexide at 70 mg/kg/day had a significantly shorter tumor latency than those treated with sulodexide at the dose of 35 mg/kg/day ( $p=0.002$ ).



There are several plausible explanations for these unexpected results: 1) Our recent in vitro study showed that HPR1 inhibitors are able to stimulate the proliferation of pancreatic cancer cells by increasing the expression of cell surface HPSGs and strengthening the FGF2 receptor-activated MAP kinase pathway (4). It is possible that sulodexide may stimulate the proliferation of PyMT-transformed breast cells in vivo by a similar mechanism; 2) HSPGs function as the co-receptor for Wnt and FGF-2, both growth factors are involved in stem cell self-renewal (8; 9). It is possible that increased cell surface HSPG levels by sulodexide may enhance the FGF signaling pathway, leading to the acceleration of breast cancer formation.

The role of HPR1 in breast tumor growth and tumorigenesis was further tested by using a genetic approach. Two miRNA constructs were prepared by using a hairpin sequence that target murine HPR1 mRNA at the nucleotide site of 746. As shown in Fig. 3, RCAS-Neu/miRNA-HPR1, were able to decrease cell surface heparan sulfate, as revealed by FACS analysis of cell surface HS. Western blot analysis confirmed the ability of these two constructs to suppress HPR1 expression in RCAS-Neu breast cancer cell lines (Fig. 3). HPR1-miRNA fragment was also cloned into the downstream of RCAS-PyMT vector. Similar results were obtained with RCAS-PyMT/HPR1-miRNA construct (data not shown). This mHPR1 microRNA insert will be subcloned into the downstream of Wnt oncogene in the avian retroviral vector (RCAS).

In vitro study showed that knockdown of HPR1 expression in RCAS-Neu cells did not affect cell proliferation (Fig. 4). We next examined the effect of HPR1 knockdown in tumor growth in a syngeneic mouse model. RCAS-Neu cells stably transfected with RCAS-Neu/HPR1-miRNA or the control construct RCAS-Neu/Ctr-miRNA were inoculated into the fat pad of FVB mice ( $5 \times 10^5$  cells per fat pad) (8-12 mice/group). Tumor growth was measured twice weekly. As shown in Fig. 5, knockdown of HPR1 expression led to a significant suppression of tumor growth in RCAS-Neu tumor cells with HPR1 knockdown.

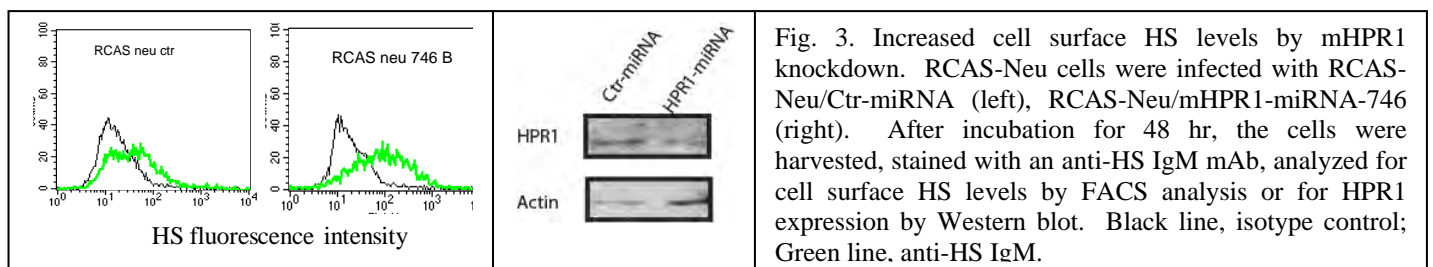
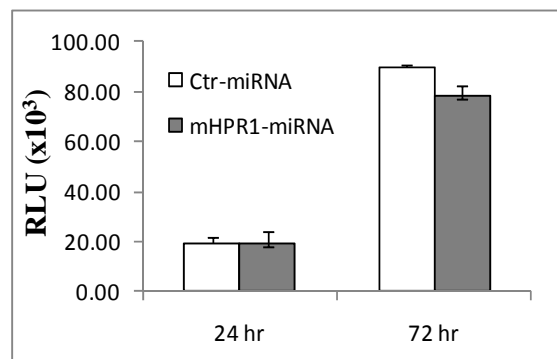


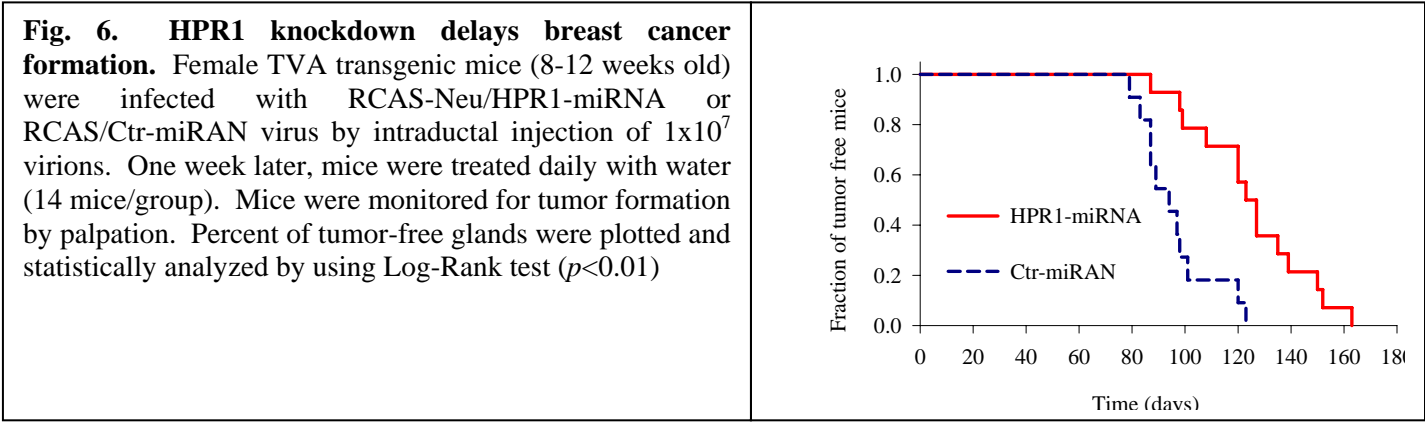
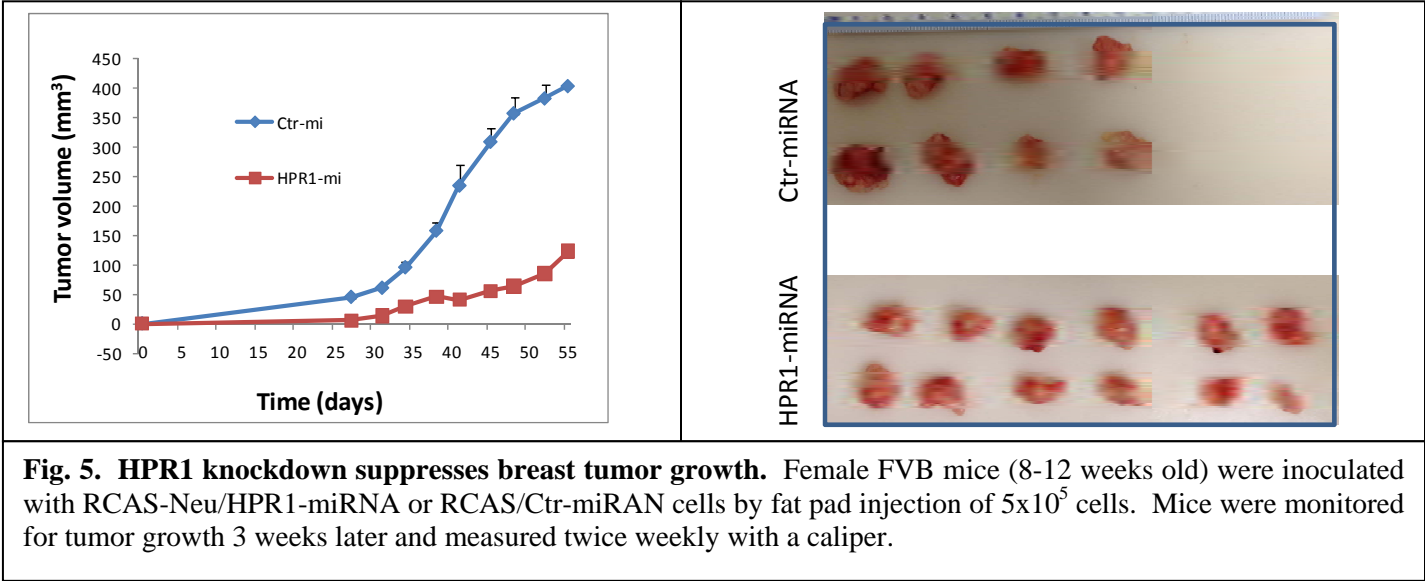
Fig. 3. Increased cell surface HS levels by mHPR1 knockdown. RCAS-Neu cells were infected with RCAS-Neu/Ctr-miRNA (left), RCAS-Neu/mHPR1-miRNA-746 (right). After incubation for 48 hr, the cells were harvested, stained with an anti-HS IgM mAb, analyzed for cell surface HS levels by FACS analysis or for HPR1 expression by Western blot. Black line, isotype control; Green line, anti-HS IgM.

**Fig. 4. HPR1 suppression does not inhibit tumor growth.** RCAS-Neu cells infected with Ctr-miRNA or HPR1-miRNA virus were seeded in 96-well plates (2000 cells/well) and incubated for 24 or 72 hr. Cell proliferation was analyzed by an ATP-based Cell-Glo assay and read in a 96-well plate reader. The data represents the mean standard deviation of one of three experiments in triplicate with similar results.



RCAS-Neu/HPR1-miRNA and RCAS-Neu/Ctr-miRNA retroviral vectors ( $1 \times 10^7$  virions/gland) were used to induce breast cancer by intraductal injection into the mammary gland of TVA transgenic mice carrying

the transgene encoding the receptor for the sub-group A avian leucosis virus. As shown in Fig. 6, mice infected with RCAS-Neu/HPR1-miRNA developed breast cancer significantly slower than those infected with RCAS-Neu/Ctr-miRNA. Taken together, our results suggest that HPR1 suppression can inhibit tumor growth and delay breast cancer formation. Since HPR1 inhibitor was able to accelerate breast tumor growth and tumorigenesis, it appears that HPR1 enzymatic activity may not contribute to its promoting effect but rather antagonize the antitumor activity mediated through its C-terminus.



# KEY RESEARCH ACCOMPLISHMENTS:

## Task 1. To determine the overall effect of HPR1 knockdown on breast tumor initiation. (Year 1)

- A. Clone the mouse HPR1 (mHPR1) and control microRNA inserts downstream of three oncogenes in RCAS vector (Q1-2) ✓
- B. Transfect DF-1 fibroblast cell line with these vectors, collect virus, and titrate the retrovirus concentration (Western blot or IF staining with anti-HA tag antibody) (Q1-2) ✓
- C. Infect breast cancer cell lines (derived from TVA transgenic mice) with these retroviral vectors, expect to their ability to suppress HPR1 expression (Analyzed by Western blot and FACS analysis of cell surface HS) ✓
- D. Breeding to produce 120 female TVA transgenic mice (Q2-3) ✓
- E. Induction of breast cancer by intraductal injection of RCAS virus (Table 1) (Q2-3) ✓
- F. Analyze cell proliferation by IHC staining for BrdU, cyclin D, c-Myc (Q3-4) ✓
- G. Analyze HPR1 expression and HS by IHC & IF staining, Western blot, FACS (Q3-4) ✓
- H. Angiogenesis analysis by quantifying the number of microvessel stained with Texas Red-conjugated dextran sulfate or IHC staining for CD31 (Q3-4) ✓
- I. Whole mount to analyze tumor initiation (Q3-4) X
- J. Gross and histological analysis of tumor metastasis in the lungs and lymph nodes (Q3-4). X

Table 1. Effect of mHPR1 knockdown on breast tumor initiation and progression induced by three oncogenes

Group	# of mice	Viral vector	Tumorigenesis	Latency	Proliferation	Angiogenesis	Metastasis
1	20	RCAS-PyMT/Ctr-miRNA	Yes	Normal	Normal	Normal	little
2	20	RCAS-PyMT/mHRR1-miRNA	?	?	?	?	?
3	20	RCAS-Neu/Ctr-miRNA	Yes	Normal	Normal	Normal	Little
4	20	RCAS-Neu/mHRR1-miRNA	Yes	Slower	?	?	?
5	20	RCAS-Wnt/Ctr-miRNA	No	No	No	No	No
6	20	RCAS-Wnt/mHRR1-miRNA	Yes?	?	?	?	?



## **REPORTABLE OUTCOMES**

1. Manuscript: Suppression of Neu-induced mammary tumorigenesis and tumor growth by heparanase knockdown in a novel somatic breast cancer model. Manuscript in preparation

## **CONCLUSION**

We have proposed a novel hypothesis that HPR1 enzymatic activity can antagonize the tumor promoting effect of the C terminus of HPR1. Data so far on a syngeneic breast cancer model and somatic mouse model suggest that knockdown of HPR1 expression causes the delay of tumorigenesis and the inhibition of tumor growth. These results suggest the overall role of HPR1 is to promote tumor growth and formation. In next fiscal year, we will determine whether the C terminus of HPR1 plays a role in promoting tumor initiation and growth, and whether the enzymatic activity of HRP1 will antagonize the tumor-stimulating effect of HPR1 mediated by its C-terminus.

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